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Brunner, S ; Hurni, S ; Herren, G ; Kalinina, O ; von Burg, S ; Zeller, S L ; Schmid, B ; Winzeler, M ; Keller, B

Abstract: Plant resistance (R) genes are highly effective to protect plants against diseases, but pathogens can overcome such genes relatively easily by adaptation. Consequently, in many cases R genes do not confer durable resistance in agricultural environments. One possible strategy to make the use of R genes more sustainable depends on the significantly improved in all lines in the greenhouse and the field, both with naturally of R genes, we overexpressed in wheat the Pm3b resistance gene against powdery mildew under control of the maize ubiquitin promoter. Four independent transgenic lines were tested in the greenhouse and the field during three years. The four lines showed a five- to 600-fold transgene overexpression compared to the expression of the endogenous Pm3b gene in the landrace "Chul". Powdery mildew resistance was morphology. The highest overexpressing line had the strongest side effects, suggesting occurring infection or after artificial inoculation. Under controlled environmental conditions, the line with the strongest overexpression of the Pm3b gene showed a dramatic increase in resistance to several independent isolates that are virulent on the endogenous Pm3b. Under a variety of field conditions, but never in the greenhouse, three of the four transgenic lines showed pleiotropic effects on spike and leaf a correlation between expression level and phenotypic changes. These results demonstrate that the successful transgenic use of R genes critically depends on achieving an optimal level of their expression, possibly in a tissue specific way.

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Transgenic *Pm3b* wheat lines show resistance to powdery mildew in the field

Susanne Brunner[1], Severine Hurni[1], Gerhard Herren[1], Olena Kalinina[2], Simone von Burg[2], Simon L. Zeller[2], Bernhard Schmid[2], Michael Winzeler[3] and Beat Keller[1,*]

[1]Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland

[2]Institute of Evolutionary Biology and Environmental Studies, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

[3]Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstrasse 191, CH-8046 Zürich, Switzerland

*For correspondence (phone +41 44 634 8230; fax +41 44 634 82 04; e-mail bkeller@botinst.uzh.ch).

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Summary

Plant resistance (*R*) genes are highly effective to protect plants against diseases, but pathogens can overcome such genes relatively easily by adaptation. Consequently, in many cases *R* genes do not confer durable resistance in agricultural environments. One possible strategy to make the use of *R* genes more sustainable depends on the modification of *R* genes followed by transformation. To test a possible transgenic use of *R* genes, we overexpressed in wheat the *Pm3b* resistance gene against powdery mildew under control of the maize ubiquitin promoter. Four independent transgenic lines were tested in the greenhouse and the field during three years. The four lines showed a five- to 600-fold transgene overexpression compared to the expression of the endogenous *Pm3b* gene in the landrace "Chul". Powdery mildew resistance was significantly improved in all lines in the greenhouse and the field, both with naturally occurring infection or after artificial inoculation. Under controlled environmental conditions, the line with the strongest overexpression of the *Pm3b* gene showed a dramatic increase in resistance to several independent isolates that are virulent on the endogenous *Pm3b*. Under a variety of field conditions, but never in the greenhouse, three of the four transgenic lines showed pleiotropic effects on spike and leaf morphology. The highest overexpressing line had the strongest side effects, suggesting a correlation between expression level and phenotypic changes. These results demonstrate that the successful transgenic use of *R* genes critically depends on achieving an optimal level of their expression, possibly in a tissue specific way.

Introduction

Wheat is the most important crop species after rice for human nutrition and has been constantly improved by breeding efforts (Feuillet et al., 2008). Important traits for wheat breeders are yield and grain quality, but the chance that wheat plants can fully develop these traits depends on their stress tolerance/resistance to biotic and abiotic factors. Therefore, wheat lines are intensively selected for disease resistance. Major disease resistance (*R*) genes have been widely used in wheat breeding for many decades because they are genetically simple and very effective. They act as immune sensors that recognise directly or indirectly pathogen-derived molecules, the effectors (Chisholm et al., 2006; Jones and Dangl, 2006). This recognition induces defence responses that limit pathogen growth (Hückelhoven, 2007; Lam et al., 2001). The majority of *R* genes confer resistance to only a subset of all races of a pathogen species, resulting in the so-called race-specific resistance. However, single major *R* genes are often rapidly overcome in lines with widespread cultivation. Therefore, breeders are constantly challenged to broaden the resistance resources in their breeding programmes.

In addition to classical breeding, several approaches have been used to modify plants for enhanced disease resistance by recombinant gene technology. Collinge et al. (2008) identified three main strategies: (i) the direct interference with pathogenicity or inhibition of pathogen physiology; (ii) the regulation of natural induced host resistance by altering the pathogen recognition or the downstream signalling; (iii) the pathogen mimicry through pathogen derived sequences. The latter strategy only applies to viral resistance and was not yet used for wheat improvement. Schlaich et al. (2006) provided the first example for an application of the first strategy in wheat that was successful under greenhouse and field conditions. Examples for the second strategy are more numerous (Altpeter et al., 2005; Anand et al., 2003b; Schweizer, 2008). To

the best of our knowledge, these engineered wheat plants were not yet tested for resistance under field conditions or were susceptible (Anand et al., 2003b). Transgenic wheat lines overexpressing the resistance gene *Lr10* showed increased resistance to *Puccinia triticina* compared to lines carrying the endogenous copy of *Lr10* in growth chamber tests and on detached leaf culture bioassays (Feuillet et al., 2003; Romeis et al., 2007). Under semi-field conditions, these transgenic plants showed a reduction of the 1000-kernel weight, suggesting that fitness costs arise as a result of the *Lr10* overexpression (Romeis et al., 2007).

R gene overexpression was also studied in other crops and in model organisms. Overexpression of *Prf* (Oldroyd and Staskawicz, 1998) and *Pto* in tomato (Tang et al., 1999), *Xa3* in rice (Cao et al., 2007) and *BAL* (*At4g16890*) (Stokes et al., 2002) in Arabidopsis resulted in enhanced disease resistance. However, it was also found that overexpression of *R* genes can result in negative effects by triggering more frequent cell death and thus might result in a yield penalty (Tang et al., 1999). In addition, the constitutive induction of defence responses might cause plant damage (Stokes et al., 2002). So far, none of these *R* gene overexpressor lines were tested in the field.

In wheat, only six *R* genes have been cloned so far, *Lr10* (Feuillet et al., 2003), *Lr21* (Huang et al., 2003), *Pm3* (Yahiaoui et al., 2004), *Lr1* (Cloutier et al., 2007), *Yr36* (Fu et al., 2009) and *Lr34* (Krattinger et al., 2009). The *Pm3* powdery mildew resistance locus occurs in seven distinct alleles (*Pm3a – g*) in the bread wheat gene pool commonly used by wheat breeding programmes. *Pm3a* contributes significantly to the powdery mildew resistance in currently grown wheat cultivars of the southern Great Plains in USA (Chen et al., 2009), while *Pm3b*, *Pm3d* and *Pm3f* are present in current Scandinavian wheat cultivars and breeding lines (Lillemo et al., 2010). The *Pm3b* allele was first identified in the landrace Chul from Uzbekistan (Briggle, 1966) and is the most common functional *Pm3* allele among 2325 tested landraces and non-elite cultivars (Bhullar et al., 2010a; Kaur et al., 2008). All seven *Pm3* alleles confer resistance to a

specific set of powdery mildew races and were molecularly isolated (Srichumpa et al., 2005; Yahiaoui et al., 2004; Yahiaoui et al., 2006). A *Pm3* allele mining strategy allowed the cloning of ten additional alleles (*Pm3k-t*) (Bhullar et al., 2009; Bhullar et al., 2010b; Yahiaoui et al., 2009).

It was previously described that *Pm3c* and *Pm3f* confer residual resistance to virulent isolates (Nass et al., 1981), and we hypothesised that this effect might be enhanced in *Pm3* overexpressor lines. Here, we describe that strongly overexpressing *Pm3b* plants showed quantitative resistance against isolates virulent on *Pm3b.* Under field conditions, powdery mildew infection was strongly reduced in four tested *Pm3b* overexpressor lines compared to the susceptible control lines and to the *Pm3b* donor line Chul. One of these transgenic lines showed no side effects, while the remaining three exhibited pleiotropic effects in the field.

Our work presented here is part of a joint project of several research groups (wheat consortium; http://www.NRP59.ch). In addition to the experiments described here, the transgenic *Pm3b* lines were also analysed in agronomical field performance tests adapted from classical variety trials in which disease resistance against several fungi, yield, yield components and kernel quality were determined. An important part of the consortium was devoted to studies of the ecological performance. For example, the transgene x environment interactions were analysed in glasshouse experiments and in the field season 2008 (Zeller et al., 2010), and the genetic and ecological consequences of introgression of transgenic wheat in a wild relative, *Aegilops cylindrica*, were studied. A set of further biosafety research experiments studied the impact of these transgenic lines on other organisms (Lindfeld et al., 2010; Peter et al., 2010; Song-Wilson et al., 2010; von Burg et al., 2010).

Results

Generation of transgenic wheat lines expressing *Pm3b*

We have transformed the powdery mildew resistance gene *Pm3b* into the susceptible wheat cv. Bobwhite SH 98 26, hence abbreviated Bobwhite, by microprojectile bombardment. T_1 offspring were tested for powdery mildew resistance by leaf segment infection tests using an isolate avirulent on *Pm3b*. Fifteen T_1 families segregated for resistance and were characterised by Southern blot analysis. Four families showed the presence of a single copy of *Pm3b*, five a full length plus one or more truncated copies of *Pm3b*, and the remaining lines had more complex transgene insertion patterns. In three families, not all transgenic plants were resistant, probably due to transgene silencing. However, in eleven of the T_1 families, presence of the transgene co-segregated with the powdery mildew resistance. All resistant plants and one susceptible, non-transgenic (null segregant) plant of these families were grown to T_2. As the null segregants (sister lines) are carrying possible (epi)genetic alterations independent of the transgene which potentially occur during the tissue culture and regeneration process, they were used as direct negative controls. Southern blot and PCR analysis showed the absence of the selectable marker gene (*manA*) in the null segregants. For further analyses, we focused on four families and named them Pm3b#1, Pm3b#2, Pm3b#3 and Pm3b#4. Southern Blot analysis suggested that Pm3b#1 to Pm3b#3 carry a single full-length copy of the *Pm3b* transgene and Pm3b#4 one full-length and one truncated copy (Figure S1; available as Supplementary material). Their corresponding sister lines were named Sb#1, Sb#2, Sb#3 and Sb#4, respectively.

Segregation analysis was performed on the T_2 families of the events Pm3b#1 to #4 and plants homozygous for the transgene loci were identified. Resistance was tested on

leaf segments of seedlings (Figure 1a), and presence of the transgene was determined by PCR analysis using *Pm3*-specific primer pairs. In heterozygous (hemizygous) families of the events Pm3b#1, Pm3b#2 and Pm3b#4, transgenes co-segregated with the resistance phenotype, and the segregation was consistent with a 3:1 resistant:susceptible ratio (Chi-square test; $P = 0.75$ for Pm3b#1, $P = 0.37$ for Pm3b#2, and $P = 0.95$ for Pm3b#4), indicating a Mendelian inheritance of *Pm3b*. In contrast, heterozygous families of event Pm3b#3 did not segregate in a 3:1 resistant:susceptible ratio (Chi-square test, $P < 0.001$), but showed a higher proportion of susceptible plants. The progeny of selfed homozygous T_2 plants of Pm3b#3 showed a phenotypical range between full susceptibility to full resistance. In total, 28% of these T_3 plants were susceptible (Table S1), although they still carried the *Pm3b* transgene as determined by PCR analysis. Southern blot analysis showed that these susceptible plants inherited the full-length transgene to at least the sixth generation (data not shown). For further propagations, only resistant plants of Pm3b#3 were used, but in all further generations a fraction of the offspring showed powdery mildew susceptibility (Table S1). When this selection of resistant parents was omitted in the T_5 and T_6 generation, which were propagated in the field, the proportion of susceptible plants increased to 44% in T_6 and to 65% in T_7. Thus, the frequency of susceptible progeny from resistant Pm3b#3 plants was similar in the greenhouse and the field.

Pm3b#2 plants show broad-spectrum resistance in the leaf segment assay

Transgenic *Pm3b* lines were infected with eight different powdery mildew isolates that are virulent on the *Pm3b* donor line Chul and on its backcross line Chul/8*Chancellor. Infection tests with these isolates on leaf segments of seedlings revealed that Pm3b#1 and Pm3b#4 were as susceptible as their corresponding sister lines and Bobwhite (Table 1 and Figure 1b). In contrast, Pm3b#2 showed lower infection levels to all tested isolates. Individual Pm3b#3 plants differed in their reactions to the same isolate, some

being susceptible and others being partially resistant. Due to the high variance in disease scores, the difference between Pm3b#3 and Sb#3 was only significant for two isolates, 97011 and 96.10. This suggests that the race-specific resistance in wheat lines with the endogenous *Pm3b* gene is broadened in Pm3b#2 as well as in a fraction of the Pm3b#3 plants to a partial resistance against isolates virulent on lines with the native *Pm3b* gene.

Transgenic *Pm3b* lines show powdery mildew resistance in the field

We tested if the powdery mildew resistance observed in leaf segment infection tests is also functional under field conditions. Previous powdery mildew virulence profiling that was performed in Switzerland in 2007 (Brunner et al., 2010) revealed that for all the *Pm3a* to *Pm3g* alleles, virulent strains are present. The virulence rate for *Pm3b* was 4% overall in Switzerland, and 7% in the Eastern part of Switzerland where the field site was situated. The powdery mildew resistance trials with the transgenic *Pm3b* lines were performed during the field seasons 2008, 2009 and 2010 in microplots, and in 2008 also in macroplots. Microplots were flanked on both sides by a row of highly susceptible wheat plants to increase powdery mildew pressure. The test lines in the microplot experiments were subjected to natural powdery mildew infection, to artificial infection, or, as control, were treated with fungicide. Cultivar Bobwhite was highly susceptible in our environment and, therefore, represents an ideal genetic background for powdery mildew resistance studies. In all three years and under natural as well as artificial infection, the transgenic *Pm3b* lines were significantly more resistant than their corresponding sister lines and the non-transformed Bobwhite parent ($P < 0.001$ for all experiments; Table 2). Interestingly, they were also more resistant than the *Pm3b* donor line Chul ($P < 0.001$ for 2009 and 2010). In the microplots 2008 and in the natural infection in 2010, Pm3b#2 was more resistant than the other transgenic *Pm3b* lines ($P = 0.027$ in 2008; $P = 0.008$ in 2010), but this effect was only marginally

significant in 2009 (P = 0.087). In 2008, Pm3b#3 was as resistant as Pm3b#1 and Pm3b#4 (P = 0.194). In fact, some plants inside the plots planted with Pm3b#3 were highly infected, but overall these plots showed low infection levels. This is in agreement with the segregation of the resistance phenotypes observed in seedling infection tests (Table S1). In 2009, we used Pm3b#3 seeds derived from T_5 plants which were not selected for resistance and therefore showed a higher susceptibility rate (44%). Consequently, Pm3b#3 was significantly less resistant than Pm3b#1 and Pm3b#2 (P = 0.002), but still more resistant than the sister line Sb#3 ($P < 0.001$; Table 2).

Expression analysis of *Pm3b* in the field

We developed a reverse transcription, quantitative real-time polymerase chain reaction (RT-qPCR) assay to quantify expression levels of the *Pm3b* gene. The specific detection of *Pm3* transcripts in this sensitive assay was challenging for several reasons. First, *Pm3* belongs to a large family of highly similar genes (Wicker et al., 2007; Yahiaoui et al., 2004 and our own unpublished data). The overall sequence homology of the members of this cluster made it difficult to identify specific primer/probe sets. The problem of a large number of highly similar genes is even more pronounced in wheat since it is hexaploid. Second, we designed primers and probes in the leucine-rich repeat (LRR) encoding region of *Pm3b* since transcript quantification assays targeting the 3' end of genes is less susceptible to artefacts due to RNA degradation and incomplete cDNA synthesis. As LRR domains are present in a number of protein classes, more sequences could potentially be co-targeted by our assay. Finally, since the wheat genome is not sequenced, *in silico* analyses on potential sequence identities are not very powerful. Since none of the qPCR assays designed by commercial software were specific or sufficiently efficient, we manually designed an assay.

We analysed the *Pm3b* expression levels of Pm3b#1 to #4 from the powdery mildew field infection tests in 2008 and 2009. RNA samples were taken first from leaves at the stem elongation stage (BBCH 32-37), when infection with powdery mildew started, and second around flowering time (BBCH 61-71), when powdery mildew infection reached its peak level. Four or three plots per line were analysed in 2008 and 2009, respectively. To obtain representative samples, leaves of three different plants from each plot were pooled. From Pm3b#3, which segregated for powdery mildew resistance, we pooled material from six different plants. In 2008, lines Pm3b#1 to #4 were analysed under natural powdery mildew infection pressure. *Pm3b* expression levels were higher at the end of flowering than at stem elongation stage ($P = 0.009$; Figure 2a). At both time points, they were similar among Pm3b#1, Pm3b#3 and Pm3b#4 ($P = 0.515$), while Pm3b#2 showed around five times higher levels ($P < 0.001$). These experiments were repeated in the following field season with the lines Pm3b#1, Pm3b#2, Pm3b#3 and the *Pm3b* donor line Chul under three different environments: natural infection, artificial powdery mildew infection and fungicide treatment. RT-qPCR results demonstrated that there was no overall effect of the treatments on the *Pm3b* expression levels ($P = 0.141$), suggesting that *Pm3b* expression was not influenced by powdery mildew infection. *Pm3b* transcript levels of the transgenic lines were significantly different from Chul ($P < 0.001$), being on average 11, 55 and 5 times higher in Pm3b#1, Pm3b#2 and Pm3b#3, respectively, compared to Chul (Figure 2b). These results were consistently found at both sampling time points with one exception: *Pm3b* expression was about ten times reduced in Chul at flowering time compared to the stem elongation stage, leading to a 130, 617 and 112 fold expression level difference to Pm3b#1, Pm3b#2 and Pm3b#3, respectively (Figure S2). This indicates a differential expression of the native *Pm3b* and the ubiquitin promoters during plant development.

To test whether the variability of resistance phenotypes between individual Pm3b#3 plants homozygous for the transgene locus correlates with differences in expression levels, we determined the *Pm3b* transcript levels of 20 individual Pm3b#3 plants and compared them to those of seven individual Pm3b#1 plants (Figure 3a). While the T_6 Pm3b#3 plants showed a wide range of *Pm3b* expression levels (between 0.01 and 3.42), expression was homogenous in Pm3b#1 (between 0.81 and 1.27; F-ratio = 27.8, $P < 0.001$). These data show that the majority of Pm3b#3 individuals show transgene silencing at different intensity, and that three of the tested plants are stronger *Pm3b* expressers than Pm3b#1. To analyse if transgene silencing causes the susceptibility observed in Pm3b#3 plants, leaf segments of seedlings were infected with powdery mildew. Resistant (no disease symptoms), intermediate resistant (20-30% of diseased leaf surface) and susceptible (60-100% of diseased leaf surface) plants were used for expression analysis. As control line, Chul/8*Chancellor was used, which carries an endogenous copy of *Pm3b* and showed no disease symptoms upon powdery mildew infection. RT-qPCR analysis showed that the resistant Pm3b#3 plants overexpressed *Pm3b* compared to Chul/8*Chancellor, while susceptible plants barely expressed the transgene, revealing transgene silencing (Figure 3b). The intermediate resistant plants showed similar *Pm3b* expression levels as Chul/8*Chancellor plants ($P = 0.470$). This suggests that similar amounts of *Pm3b* transcripts lead to a less complete protection against powdery mildew in the Bobwhite genetic background than in the Chancellor genetic background.

Pm3b#2, Pm3b#3 and Pm3b#4 show pleiotropic phenotypes under field conditions

When the Pm3b#1 to #4 lines were grown in growth chambers or in the greenhouse, no pleiotropic phenotypes were observed. However, in all three field seasons, leaves of Pm3b#2 and Pm3b#3 turned light green or even yellow (Figure 4a). The chlorosis was

not observed in developing leaves, but increased with age in fully grown leaves. All Pm3b#2 plants were affected, while Pm3b#3 plants segregated for this phenotype. Leaf chlorophyll content measured at the end of flowering (BBCH 69) in flag leaves was reduced by 15% in Pm3b#2 and by 8% in Pm3b#3 compared to their sister lines (Table 3), but was most likely even lower at later time points. The stomatal conductance (g_s) was also reduced by 21% in Pm3b#2 (245 ± 15.8 mmol $m^{-2}s^{-1}$) compared to Bobwhite (312 ± 17.4 mmol $m^{-2}s^{-1}$) and Pm3b#1 (298 ± 12.3 mmol $m^{-2}s^{-1}$; P = 0.002). In Pm3b#2 and Pm3b#3, leaf thickness (as deduced from the measurement of the surface area and fresh weight of single flag leaves) was not altered, indicating that the brighter leaf colour resulted from a lower chlorophyll concentration (data not shown). Considering the high expression level of Pm3b#2 and the positive correlation between the powdery mildew resistance level and the *Pm3b* transgene expression level in Pm3b#3, strong *Pm3b* overexpression in Pm3b#2 and in a fraction of Pm3b#3 possibly caused this leaf damage. Interestingly, we observed that chlorotic Pm3b#3 plants showed less growth of powdery mildew, supporting this hypothesis.

Pm3b#2 had a reduced plant height compared to its corresponding sister line. This difference occurred in all three years (P < 0.001; Table 3 and S2). Besides the genotype, also the treatment influenced the plant height, as fungicide treated plants were taller than those without fungicide in 2009 (P = 0.004). The same trend was also observed in 2008 (P < 0.001), but this difference could also be due to a block effect. In 2010, plant height was not different between the treatments (P = 0.143). Inspection of the individual wheat lines indicated that the overall plant height difference between the treatments in 2009 was actually due to a reduced plant height of the susceptible lines (sister lines and Bobwhite) in the infected plots compared to the fungicide treated plots (P = 0.001). The resistant *Pm3b* lines had the same height in all three treatments (P = 0.478).

A fraction of the Pm3b#2 and Pm3b#4 plants had flowers that remained open after flowering, a phenotype known from male sterile plants (Figure 4b). Indeed, we detected many flowers that did not produce seeds. The overall seed set rates of Pm3b#2 and Pm3b#4 were reduced compared to their corresponding sister lines in microplots in 2008 (Table 3). For Pm3b#4 this effect was significant ($P = 0.048$), for Pm3b#2 it was marginally significant ($P = 0.070$). In the macroplots 2009 and 2010, this reduction was again observed in Pm3b#4 ($P < 0.001$), but not in Pm3b#2 ($P = 0.573$ in 2009, $P = 0.298$ in 2010; Table S2), indicating that seed set reduction depends on subtle environmental differences. This is also supported by the fact that in 2009, the flower phenotype was not observed in the microplots of the powdery mildew infection trials, but in the propagation plots that were on the same field (data not shown).

In the first two field seasons, we monitored the phenological stages at seven or more time points throughout the growing season. We could not detect any consistent difference in developmental stages between the transgenic and the corresponding sister lines (data not shown).

Among the traits described above, we found no consistent difference between the sister lines and Bobwhite. For example, Sb#3 was shorter and Sb#4 was taller compared to the other sister lines ($P = 0.038$ for both; Table S2). However, this difference was not found in 2009 (Table 3). Possibly, these effects are due to differences in seed properties, as the seeds for 2008 were grown in different greenhouse chambers in different seasons, while the seeds of 2009 were all propagated in the same field in 2008. Together, this indicated that tissue culture induced effects played a minor role compared to the effects induced by the transgene.

Discussion

The *Pm3b* transgene confers resistance under field conditions

The four transgenic lines Pm3b#1 to #4 were more resistant than the sister lines and the non-transformed Bobwhite plants in all leaf segment infection tests using isolates avirulent on *Pm3b* (Figure 1a). This increased resistance was also observed in field environments which have complex mixtures of avirulent and virulent powdery mildew races (Table 2). Interestingly, all transgenic *Pm3b* lines were more resistant than the *Pm3b* containing landrace Chul. The overexpression of *Pm3b* in the transgenic lines is possibly responsible for the increased resistance. The high powdery mildew susceptibility of the parental cultivar Bobwhite and the sister lines suggests that they have no seedling or adult resistance and that the observed resistance in the Pm3b#1 to #4 lines is solely due to the *Pm3b* transgene. However, it is also known that the effectiveness of *R* gene function can depend on the genetic background of the breeding material (Cao et al., 2007). Thus, we can not exclude that cultivar Bobwhite simply has a more efficient signal transduction mechanism that is triggered by *Pm3b*-mediated recognition.

In the field resistance tests, Pm3b#2 showed the lowest infection levels compared to the other lines, most likely due to the additional quantitative resistance against races virulent on *Pm3b* observed in leaf segment infection tests (Table 1, Figure 1b). Several studies have reported that *R* gene overexpression induces enhanced resistance (Cao et al., 2007; Feuillet et al., 2003; Oldroyd and Staskawicz, 1998; Stokes et al., 2002; Tang et al., 1999). Field trials with potato transformed with the late blight resistance gene *RB* under control of the native promoter revealed a positive correlation between *RB* transcript abundance and the resistance level (Bradeen et al., 2009). Bieri et al. (2004) have shown that the level of *R* gene product is a critical parameter for resistance gene function. In addition, the gene-dosage dependent action of many *R*

genes with different reaction patterns in homozygous vs. heterozygous plants indicates that the amount of *R* gene product is critical. The mechanism by which *R* gene overexpression leads to enhanced resistance is not elucidated yet. It was hypothesised that R proteins may have some intrinsic activity and their overexpression could result in protein levels that suffice to induce detectable defence responses even in the absence of the matching avirulence genes. Abundant R proteins could also titrate proteins that normally repress R protein activation (Frost et al., 2004; Oldroyd and Staskawicz, 1998; Stokes et al., 2002).

Transgene expression and silencing are not affected in field environments

Quantitative transgene expression analysis of field grown T_4 and T_5 plants showed that *Pm3b* expression levels were very similar in 2008 and 2009 and with or without powdery mildew infection (Figure 2). This demonstrates that transgene expression shows little dependence on the environment. Our data suggest that the frequency of transgene silencing in line Pm3b#3 under field conditions followed the same trend as in the greenhouse. The random silencing of only individual, but not all plants of a Pm3b#3 generation has also been reported for two other transgenic wheat lines where the occurrence of transgene silencing was positively correlated with increased levels of transgene methylation (Anand et al., 2003a; Howarth et al., 2005). Transgene silencing is a well known phenomenon in the transformation of plants (Vaucheret et al., 1998), including wheat (Alvarez et al., 2000; Anand et al., 2003a; Bourdon et al., 2002; Chen et al., 1998; Demeke et al., 1999; Howarth et al., 2005; Iyer et al., 2000; Li et al., 2005; Müller et al., 1996). Unstable transgene expression is known to occur more frequently in transformants carrying complex insertion patterns with multiple transgene copies (Vaucheret et al., 1998). However, there are also reports of silenced transgenes that occur as single-copy (Elmayan and Vaucheret, 1996), as was the case for *Pm3b*.

One of four lines showed no pleiotropic phenotypes

We did not observe any pleiotropic phenotypes under the controlled conditions of greenhouses or growth chambers during seed propagation. Under field conditions, however, an altered leaf phenotype and fertility problems occurred. Overexpression of the tomato CC-NB-LRR-type of resistance gene *Prf* (Oldroyd and Staskawicz, 1998) and the rice *Xa3*, an LRR-receptor kinase-like *R* gene (Cao et al., 2007), led to broad-spectrum or enhanced resistance, respectively, without any deleterious side effects. Wheat lines overexpressing the CC-NB-LRR encoding *Lr10* gene were less diseased than the *Lr10* donor line, and no pleiotropic phenotypes were observed except for a reduced grain weight under semi-field conditions (Feuillet et al., 2003; Romeis et al., 2007). The overexpression of tomato *Pto*, which encodes a serine/threonine kinase (Tang et al., 1999) and *BAL*, an NBS-LRR R protein from Arabidopsis (Stokes et al., 2002), also conferred enhanced resistance. However, these lines exhibited microscopic lesions, or dwarfing and twisted leaves, respectively. These phenotypes are different from the pleiotropic effects in the Pm3b#2, Pm3b#3 and Pm3b#4 lines. Interestingly, however, the phenotypes observed in our study have often been observed in wheat breeding programmes. For example, chlorosis and reduced fertility were observed in the wheat pre-breeding programme of ICARDA using wild wheat progenitors (Valkoun, 2001).

The molecular basis of the pleiotropic phenotypes in Pm3b#2, Pm3b#3 and Pm3b#4 is not known. Since the corresponding sister lines were not affected, the observed effects must be due to the *Pm3b* transgene. The fact that fertility problems and chlorosis both occurred in two independent events suggests that the *Pm3b* gene product and not transgene position effects, for example the disruption of endogenous genes, cause the pleiotropic effects. The co-occurrence of strong *Pm3b* expression, strong powdery mildew resistance and chlorotic leaves in a segregating subset of Pm3b#3 plants demonstrates that at least the chlorosis is a consequence of the *Pm3b* gene product. In

our lines, the *Pm3b* transgene was under the control of the maize ubiquitin promoter. This promoter was reported to be strongly and constitutively active in all tissues (leaves, roots, flowers, immature inflorescence, endosperm and embryo) (Jones and Sparks, 2009; Rooke et al., 2000). Thus, it is possible that the ectopic expression of *Pm3b* causes pleiotropic phenotypes. However, not all transgenic *Pm3b* lines showed all pleiotropic effects and ectopic *Pm3b* expression can not be the only determinant of these alterations. Since Pm3b#2, which is the strongest *Pm3b* expresser, shows all side effects, strong overexpression possibly caused the observed side effects. However, this simple *Pm3b* transcript dose effect can not explain why Pm3b#1 shows no side effects and Pm3b#4 has reduced fertility, while both have similar *Pm3b* expression levels. In a minority of independent transgenic wheat lines, the ubiquitin promoter driven expression was shown to be restricted to only a few tissues (Rooke et al., 2000), suggesting that also differences in expression levels between different tissues may exist. Since reduced fertility can be caused by many conditions (high and low temperatures, water stress, high humidity, boron deficiency and low radiation (Subedi et al., 1998 and references therein)), a very high PM3B protein content might interfere with any of a number of cellular processes. Transcriptome and metabolome profiling of specific tissues may help to identify candidate pathways that are affected by the overexpression or ectopic expression of *Pm3b*. Such analyses have shown to be very sensitive and are used for the assessment of possible adverse effects of transgene expression (Barros et al., 2010; Kogel et al., 2010).

Although new natural and synthetic promoters have been described (Furtado et al., 2009; Rushton et al., 2002), they still need to be validated under field conditions. In fact, the ubiquitin promoter is the only promoter that was widely used in GM wheat grown in the field. Since powdery mildew attacks only the epidermis of the aerial parts of wheat plants, a promoter that is only active there would be optimal. In fact, the wheat GstA1 promoter fulfils theses criteria and was studied intensively in wheat plants

(Altpeter et al., 2005). Therefore, this promoter represents an ideal tool to test if tissue-specific expression of *Pm3b* will prevent pleiotropic effects while still conferring powdery mildew resistance.

The sustainable use of *Pm3* alleles as transgenes

Since elite wheat cultivars represent only a small fraction of the wheat gene pool, favourable alleles, genes or gene complexes from landraces and wild relatives are used in breeding (Baum et al., 1992; Feuillet et al., 2008). It was suggested that the natural diversity at *R* gene loci should be better exploited (Hammond-Kosack and Parker, 2003). However, undesirable gene linkages (linkage drag), and in the case of the use of wheat progenitors also the occurrence of fertility problems and strong hybrid necrosis, are limiting the use of valuable genes (Valkoun, 2001). Our study shows that the transgenic (or cisgenic if the native promoter is used) use of an *R* gene offers a possibility to circumvent this problem. A *Pm3* allele mining strategy showed that the genetic resources indeed can be accessed at the molecular level (Bhullar et al., 2009).

To improve the durability of major *R* genes, several strategies in breeding and agriculture were developed (McDonald and Linde, 2002). Based on the characteristics of the pathogen, it was suggested that the multiline strategy is most promising for the wheat – powdery mildew pathosystem (McDonald and Linde, 2002). Multilines are mixtures of different lines of ideally identical genetic background, each carrying a different *R* gene introgressed by backcrossing into the recurrent parent (Mundt, 2002; for reviews see Wolfe, 1985). They have been proven effective in disease reduction in small grain crops (reviewed in Mundt, 2002; Zhu et al., 2000). The transformation of wheat with different, single *Pm3* alleles each will allow the production of *Pm3* multilines which will be genetically identical except for the *Pm3* allele.

A second frequently discussed and partially applied strategy is the pyramiding of resistance genes. In this strategy, several different *R* genes are combined in the same

plant, making the adaptation of the pathogen more difficult (McDonald and Linde, 2002). The transgenic use of *Pm3* alleles would allow the stable combination of different *Pm3* alleles in the same line which is not possible by classical breeding.

It is commonly known among breeders that greenhouse studies do not unravel the agronomical potential of a line. Not surprisingly, our results are showing the necessity of field trials to evaluate agronomically relevant field resistance. Field trials are also important for basic research, as field conditions might reveal aspects on the gene function that are not detectable under controlled conditions. By only using four independent transgenic *Pm3b* lines, we detected different pleiotropic effects. Blechl and Jones (2009) suggested that 8-10 events may be required for functional genomics research, 20 or more for applied research and hundreds of different transgenic events for commercial application. In the frame of the current legislation in Switzerland and the EU, it is very challenging to obtain the required legal permits for field releases and it is currently not feasible within regular research projects to work on large numbers of events.

Our work shows a promising first step in the transgenic use of an *R* gene. The further development of this work for integration into breeding would require the testing of a large number of events, the use of more specific promoters and the creation of multilines with different *Pm3* alleles.

Experimental procedures

Plasmid constructs used for wheat transformation

A 4.5-kb fragment containing the entire coding region of *Pm3b* (Yahiaoui et al., 2004) was amplified from genomic DNA by PCR using primers UP6 (5'-GGCACAGACAAAG-CTCTG-3') and N3'SP3R (5'-ACAATCAGGGATCAGGCC-3') (Srichumpa et al., 2005) and nested primers BamHI-1 (5'-TTAATTGGATCCCAATGGCAGAGCGGGTGGTC-3') and BamHI-2 (5'-TATATAGGATCCGCTTCAGCTCCGGCAGGCCTG-3'). The *Bam*HI-fragment of the amplicon was cloned under the control of the maize ubiquitin (*Ubi*) promoter (ubi) with the nopaline synthase (*Nos*) terminator (nos) into the *Bam*HI site of vector pAHC17 (Christensen and Quail, 1996) and verified by sequencing. For transformation, only the *Pm3b* gene cassette (*ubi:Pm3b*) was used, which was released from the vector backbone by enzymatic digestion using *Not*I restriction sites previously introduced into pAHC17 5' of ubi and 3' of nos (S. Travella, unpublished). Similarly, the selectable marker gene phosphomannose isomerase (Pmi; Reed et al., 2001) was cloned under the control of the maize ubiquitin promoter into pAHC17 (S. Travella, unpublished). Prior to bombardment, the gene cassette (*ubi:Pmi*) was enzymatically released from the vector backbone using a *Hind*III and a *Not*I site, the latter being previously introduced 3' of nos.

Biolistic transformation of the susceptible wheat Bobwhite SH 98 26

The hexaploid spring wheat cultivar Bobwhite SH 98 26, which was developed by CIMMYT in Mexico, was transformed via particle bombardment essentially as described (Pellegrineschi et al., 2002). In summary, 3,000 immature wheat embryos were isolated from freshly harvested wheat seeds and co-transformed in seven separate experiments with the *Pmi* and the *Pm3b* gene cassettes by particle

bombardment. Primary transformants (T_0 plants) were regenerated in tissue culture and selected on mannose containing media (Wright et al., 2001). Twenty-five of them carried at least one full length copy of *Pm3b* as inferred from Southern blot analysis and were allowed to self.

Genotypic characterization of wheat plants transgenic for the *Pm3b* gene

Transgenic T_0 and T_1 plants were analyzed by DNA gel blots. Isolation of genomic DNA from leaves and Southern hybridization were performed as described (Stein et al., 2001; Travella et al., 2006). DNA was digested with restriction enzymes *Bam*HI and *Sac*I. Cleavage with *Bam*HI produces a 4.5-kb fragment containing the complete coding region of *Pm3b*, and *Sac*I cuts once inside the gene cassette, allowing determination of insert copy number. Membranes were hybridized with probes R081 or SuB3 that bind to PM3B encoding sequences. R081 and SuB3 were amplified by PCR from the *Pm3b* containing plasmid used for transformation with the primer pair R081A (5'-TGGGCTCCAGAAACCAGA-3') and R081B (5'-TTTGATGCTGCCCAGTTG-3') or Cint18F (5'-CCAATACTCAAGCTTAGCTACAA-3') and Cint17R (5'-GTGGCAGAGGG-AAGTCAT-3'), respectively. Absence of co-transformed contaminations of the gene cassettes with plasmid backbone containing beta-lactamase (*bla*) was confirmed in events Pm3b#1 to #4 by PCR using primers Amp-F1 (5'-TTTCCGTGTCGCCCTTA-TTC-3') and Amp-R1 (5'-CAGTGAGGCACCTATCTCAG-3') with the parameters: 34 cycles of 30 s at 94°C, 30 s at 53°C, 1 min at 72°C, and by Southern blot analysis using the full length *bla* gene as probe (816 bp).

For segregation analysis, presence of the transgene was determined by PCR with the primer pairs SuB13 (5'-TGCCTAGAAGATCTATGCTTATCAG-3') and SuB8 (5'-CCG-CTCACGGACTAGCCTC-3'), or sbi342 (5'-TGGGCAGCATCAAACGC-3') and sbi143 (5'-CAAGACCGGCAACAGGATTC-3'). PCR using the primer pair SuB13/SuB8 was performed with the following parameters: 30 cycles of 30 s at 94°C, 30 s at 65°C, 50 s

at 72°C. The parameters for the primer pair sbi342/sbi143 were 30 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C.

RNA isolation and cDNA synthesis

For expression analysis of *Pm3b* in field grown plants, a 4 cm leaf segment of the penultimate fully developed leaf from three plants per plot at stem elongation stage (BBCH 32-37) or of the flag leaf from three plants per plot at flowering stage or a few days later (BBCH 61-71) were pooled. For Pm3b#3, either leaf material from 6 plants per plot was pooled, or each leaf piece was analysed individually. At each time point, leaf material was harvested from four (2008) and three (2009) plots per line. From greenhouse grown Pm3b#3 plants, 3 cm segments of the second and third leaf were pooled. Leaf samples were frozen immediately after harvesting and stored at -80°C.

Leaf material was ground in custom-made polyethylene (PE) bags in the presence of 2 ml RNA lysis buffer (4 M guanidine isothiocyanate, 10 mM Tris pH 7.5, 0.97% β-mercaptoethanol) using a hand homogeniser as described for DNA extraction (Stein et al., 2001). Per sample, 190 µl of the lysate was purified with the SV Total RNA Isolation System kit (Z3100, Promega, Dübendorf, Switzerland) including DNase I treatment. RNA quantity and purity was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, USA). Only RNA samples with A260/A280 ratios between 2.1 and 2.2 were further processed. RNA integrity was checked by electrophoretic separation on a 1% agarose gel in 1x TAE running buffer (40 mM Tris-acetate, 2 mM EDTA). First strand cDNA was synthesised from 500 ng of total RNA using 40 units of M-MLV Reverse Transcriptase RNAse H^- Point Mutant (9PIM368, Promega, Dübendorf, Switzerland), 2.5 µM Oligo$(dT)_{20}$, 0.5 mM dNTPs, 1x M-MLV-RT reaction buffer in a total volume of 12 µl, and incubation times und temperature were adopted from the manufacturer's protocol. After reverse

transcription, cDNA was treated with 2.5 units of RNAse H (M0297, New England Biolabs Inc., Ipswich, USA) at 37°C for 20 minutes and inactivated at 65° C for 20 minutes. To check for genomic DNA contamination, RT-minus controls were incorporated in the RT-qPCR experiments (see below) for the target gene *Pm3b* and the reference gene *GAPDH* and, but not for Ta2776 and Ta54227 since the corresponding probes only hybridise on cDNA. All quantification cycle (Cq) values of the RT-minus controls were at least 5 cycles above the corresponding RT-plus, thus above the lower limit recommended by Bustin and Nolan (2004).

_Pm3b_ expression analyses by RT-qPCR

Primers and probes were designed using Primer Express® Software v2.0 (Applied Biosystems, Rotkreuz, Switzerland) or the online tool GenScript Real-time PCR (TaqMan) Primer Design (https://www.genscript.com/ssl-bin/app/primer) or manually. Primer pairs were *in silico* validated for specificity using BLAST (http://blast.ncbi.nlm.nih.gov). The selected primer / probe pair for the target gene *Pm3b* was shown to be specific to all known *Pm3* alleles. PCR products of the four primers pairs (Table 4) were cloned and sequenced, confirming that only the targeted sequences were amplified. Primers and probe concentrations were optimised as described in the TaqMan® Universal PCR Master Mix protocol (430449, Applied Biosystems) and are indicated in Table 4. Absence of primer dimers was confirmed by examination of dissociation curves with the Applied Biosystems 7500 Software (version 2.0.2.). PCR efficiency was determined on the sample Pm3b#1 using calibration curves based on six serial dilutions $\log_{10}$ for each amplicon. All primer/probe sets used for the reference genes had 97-102% PCR efficiencies. For the target gene *Pm3b*, efficiency was determined for each experiment and time point separately (macroplots 2008 93% (stem elongation) and 94% (flowering), microplots 2009 92% (stem elongation) and 90% (flowering), Pm3b#3 greenhouse 99%, Pm3b#3/Pm3b#1 field 102%).

As reference gene candidates, we used GAPDH (Travella et al., 2006), Ta.2776 and Ta.54227 which were shown to be stably expressed in wheat (Paolacci et al., 2009). Reference gene validation was performed with a subset of eight samples (from microplots 2009) using geNorm, a Visual Basic Application for Microsoft Excel (Vandesompele et al., 2002). As pairwise variation was below the cut-off value of 0.15 for all samples, no further candidate reference genes were tested. In the complete dataset, $V_{2/3}$ were equal to 0.138 (macroplots field 2008, Figure 2a), 0.107 (microplots field 2009, Figures 2b and S2), 0.198 (Pm3b#1 and Pm3b#3, field; Figure 3a) and 0.120 (Pm3b#3 greenhouse, Figure 3b). Data normalisation was performed as described in Hellemans et al. (2007).

MGB hydrolysis probes (TaqMan®, Applied Biosystems, Rotkreuz, Switzerland) were used for *Pm3b* (*Ta.31015*), *Ta.2776* and *Ta.54227* (Table 4). For GAPDH (*Ta.30768*), CYBR® green (4309155, Applied Biosystems) was used. The RT-qPCR reaction was carried out using 4μl of eight fold diluted cDNA in a 16 μl reaction volume containing 8 μl PCR Master Mix (4309155 for CYBR® green, 4304437 for probes, Applied Biosystems) in optical 96-well plates (4346906, Applied Biosystems). RT-PCR was carried out in an Applied Biosystems 7500 Real-Time PCR System using manufacturer's default cycling conditions (50℃ for 20 s, 95℃ for 10 min followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min). In all experiments, at least three biological replicates of each sample were tested, and reactions were carried out with three (*Pm3b*) or two (reference genes) technical replicates. Technical replicates with standard deviation of C_q values above 0.5 were excluded from further analysis. As not all samples could be analysed on a single plate, samples were arranged in a randomized block design maximising the target genes per plate. Variation between interrun calibrators from the different plates was in the range of the technical replicates within the plates. Therefore, interrun calibration was omitted.

Leaf segment infection tests with powdery mildew

For powdery mildew infection tests on leaf segments, plants were raised in a growth chamber at 20°C/16°C day/night temperature with 70% relative humidity for ten days. Powdery mildew isolates 96236, 96224, 07230 or 07296, which are avirulent on the *Pm3b* donor line Chul and its backcross line Chul/8*Chancellor, were used for screening for resistant lines and for segregation analysis. Segments of the primary leaf were placed on 0.5% phytoagar (supplemented with 30 ppm benzimidazole) plates, infected with freshly propagated conidiospores (Winzeler et al., 1991), incubated at 20°C with 16h light per day and 80% relative humidity, and scored after 8 days as previously described (Kaur et al., 2008). The powdery mildew strains used in this study originate from the former collection of French isolates at INRA Rennes (France), or are Swiss isolates from the former isolate collection of ART Reckenholz (Switzerland) and our own collection (Brunner et al., 2010; Ph. Streckeisen, unpublished data).

Statistical Analysis

We used the statistical software GenStat (VSN International Ldt.) to fit multiple linear regression models for all variables. Residual plots were examined to identify outliers and to check if the assumptions of normality and homoscedasticity were fulfilled. Data were analysed untransformed except for the data of expression levels in 2009 which were log transformed to meet model assumptions. The critical significance level was 0.05 in all analyses.

Field testing

Field experiments were carried out at the Agroscope Reckenholz-Tänikon Research Station (ART) in Zürich-Reckenholz, Switzerland. The trials were sown on 30.3.2008, 19.3.2009 and on 25.3.2010. Per microplot (1.0 x 1.3 m), 200 (2008) or 300 (2009, 2010) viable seeds were sown in five rows using a Hege 90 Seedmatic seeding

machine (Hege Maschinen, Eging am See, Germany). In macroplots (1.08 x 3 m in 2008 and 2009, 1.08 x 4 m in 2010), 400 viable seeds per m^2 were sown in six rows using an Oyjord plot drill system (Wintersteiger AG, Ried, Austria). In February, the mineralised Nitrogen in the top 100 cm of soil was 49.8 kg N ha^{-1} (2008), 47.6 kg N ha^{-1} (2009) and 41.7 kg N ha^{-1} (2010). Nitrogen fertilizer (30 kg N ha^{-1}) was applied at the first leaf stage (phenological stage BBCH 11; Lancashire et al., 1991) and at the flag leaf stage (BBCH 39) in 2008, before sowing and at tillering (BBCH 22-29) in 2009 and immediately after sowing and at the beginning of tillering (BBCH 14-22). Conventional application of herbicide and insecticide was performed. Note that in 2008, no insecticide was applied in the macroplots to allow biosafety research projects observing insect food webs.

Macroplots were arranged in a randomised complete block design (2008) or in a latin square design with incomplete sub-squares (2009, 2010), respectively, with four replications. Microplots were arranged in a randomized split plot design with four replications. The lines were randomly allocated to plots within each block, with one plot of each line per block. The three treatments, natural powdery mildew infection (in two complete replicas in 2008), artificial powdery mildew infection and fungicide treatment were arranged in blocks in 2008, and randomised in 2009 and 2010. Treatments were separated with a 5.3 m (2008), a 3.96 m (2009) and a 2.64 m (2010) border crop of spring triticale cv. Trado. To increase the powdery mildew disease pressure, all tracks of experimental microplots were flanked on both sides at a distance of 0.25 m with a spreader row, consisting of a 9:1 mixture of the susceptible varieties FAL94632 and Kanzler. For the fungicide treatment, experimental plots and spreader rows were treated two (2008) and three (2009, 2010) times with Prosper (500g l^{-1} Spiroxamine; Leu + Gygax AG, Birmenstorf, Switzerland) between May and June. For artificial powdery mildew infection, seedlings of wheat cv. Kanzler were grown in Jiffy pots (Jiffy Products GmbH, Mölln, Germany) in the greenhouse and infected with powdery mildew

isolate 96224 which is avirulent on *Pm3b*. These pots were planted into the spreader rows every 1.0 m (2008) and every 0.8 m (2009, 2010) in April. In June 2009, the planting of infected pots was repeated, as the warm and dry weather inhibited proper powdery mildew disease development and spreading.

Powdery mildew disease symptoms were scored on the basis of a 1 to 9 scale as described by Kmecl et al. (1995). Each year, plots were scored weekly from end of May to mid of June during four subsequent weeks and a last time end of June. AUDPC was calculated as described (Jeger and Viljanen-Rollinson, 2001; Shaner and Finney, 1977). Leaf chlorophyll content was estimated on flag leaves in June 2008 using a portable chlorophyll meter (SPAD 502, Minolta, Osaka, Japan) and leaf area was measured with a LI-3100 Area Meter (LI-COR, Lincoln, NE, USA). Stomatal conductance of sun exposed green leaves (lower side) was measured at the milk stage (BBCH 75) using a SC-1 Porometer (Decagon Devices, Pullmann, NE, USA). Plant height was measured from the soil surface to the top of the ear excluding the awns. Seed set was determined on the main shoot of five plants per plot.

The whole experiment was surrounded by a 2.6-m guard strip of the tall spring triticale cv. Trado. The experimental plots were netted during germination and grain filling. On 13.6.2008 the field experiment was partially damaged by vandals by cutting off the plant heads with sickles. After this incident, all samplings, scorings and measurements were restricted to undamaged rows.

Consent to release these transgenic lines (application B07002) was obtained from the Swiss Federal Office for Environment under the Release Ordinance of 25.8.1999 (Nr. 814.911 in Federal Legislation) and the Gene Technology Act (Federal Law on Non-Human Gene Technology of 21.3.2003; Nr. 814.91 in Federal Legislation) in compliance with the draft of the revised Release Ordinance of 21.11.2005 and the EU Directive 2001/18/EC. Each line (transgenic event) was described in detail and was approved individually.

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Supporting Information

Table S1 Powdery mildew susceptibility analysis of Pm3b#3 seedlings by leaf segment infection tests.

Table S2 Morphological traits of transgenic Pm3b lines (mean ± SE).

Figure S1 Southern Blot analysis of Pm3b#1-4 and Sb#1-4.

Figure S2 Relative *Pm3b* mRNA levels in landrace Chul and transgenic *Pm3b* lines at flowering stage in microplots in 2009.

Tables

Table 1 Infection of control and transgenic *Pm3b* seedlings with powdery mildew isolates virulent on wheat lines with an endogenous *Pm3b* gene. Disease was scored as percentage of the surface area of tested leaf segments that was infected with powdery mildew (mean of three replicates ± SE).

	Isolate								
Line	07002	07004	07226	07227	97011	98229	DB	96.10	07296[1]
Bobwhite	75 ± 5	77 ± 3	85 ± 5	87 ± 3	70 ± 6	87 ± 7	n.d.	73 ± 3	70 ± 10
Chul	73 ± 3	73 ± 7	83 ± 7	83 ± 3	73 ± 9	83 ± 9	60 ± 12	80 ± 6	5 ± 3
Chul/8*CC	83 ± 3	77 ± 3	83 ± 7	83 ± 3	77 ± 3	97 ± 3	70 ± 6	80 ± 6	7 ± 3**
Chancellor	80 ± 6	70 ± 6	87 ± 3	87 ± 3	n.d.	93 ± 7	73 ± 7	77 ± 3	63 ± 7
Pm3b#1	70 ± 6	60 ± 10	87 ± 3	73 ± 3*	63 ± 7	67 ± 3	40 ± 15	73 ± 3	0 ***
Sb#1	73 ± 7	77 ± 9	80 ± 6	87 ± 3	60 ± 10	83 ± 7	57 ± 13	57 ± 15	83 ± 7
Pm3b#2	47 ± 7*	18 ± 2***	43 ± 9*	42 ± 14*	15 ± 3***	12 ± 4**	7 ± 2***	18 ± 7**	0 ***
Sb#2	77 ± 3	73 ± 3	80 ± 6	83 ± 3	77 ± 3	90 ± 10	70 ± 0	77 ± 3	87 ± 3
Pm3b#3	73 ± 17	73 ± 3	60 ± 12	83 ± 3	53 ± 3**	70 ± 21	37 ± 15	37 ± 12*	8 ± 3***
Sb#3	83 ± 7	83 ± 3	87 ± 3	80 ± 6	83 ± 3	93 ± 7	70 ± 6	73 ± 3	87 ± 3
Pm3 #4	83 ± 7	77 ± 3	87 ± 3	83 ± 3	77 ± 3	93 ± 7	67 ± 3	77 ± 3	0 ***
Sb#4	77 ± 3	87 ± 3	87 ± 3	70 ± 12	73 ± 3	93 ± 7	63 ± 3	67 ± 9	78 ± 2

[1]Isolate 07296 was used as avirulent control
Chul/8*CC was compared to Chancellor and the transgenic lines Pm3b#1-4 to their respective sister lines; statistical significance was tested using Student's T-tests (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$); SE; standard error

Table 2 Field evaluation of *Pm3b* transgenic and control lines for powdery mildew resistance. Values report the area under the disease progress curve (AUDPC) (mean of four replicates ± standard error).

		Microplots		Macroplots
Year	Wheat line[1]	Natural[2]	Artificial[3]	Natural[2]
2008	Bobwhite	161 ± 7	188 ± 3	53 ± 23
	T4 Pm3b#1	40 ± 9	11 ± 5	0
	T4 Pm3b#2	15 ± 5	6 ± 2	0
	T5 Pm3b#3	36 ± 10	21 ± 14	0.1 ± 0.1
	T4 Pm3b#4	46 ± 9	19 ± 8	0.5 ± 0.5
	T4 Sb#1	168 ± 5	195 ± 2	105 ± 5
	T4 Sb#2	166 ± 4	190 ± 5	84 ± 16
	T4 Sb#3	171 ± 4	191 ± 2	86 ± 29
	T4 Sb#4	171 ± 3	179 ± 9	124 ± 12
2009	Bobwhite	227 ± 8	229 ± 11	
	Chul (*Pm3b*)	157 ± 8	161 ± 14	
	T5 Pm3b#1	84 ± 19	96 ± 11	
	T5 Pm3b#2	81 ± 17	80 ± 16	
	T6 Pm3b#3	139 ± 18	110 ± 15	
	T5 Sb#1	240 ± 9	239 ± 5	
	T5 Sb#2	230 ± 16	232 ± 11	
	T5 Sb#3	238 ± 10	229 ± 10	
2010	Bobwhite	221 ± 6	210 ± 8	
	Chul (*Pm3b*)	186 ± 4	146 ± 14	
	T5 Pm3b#1	39 ± 18	0	
	T5 Pm3b#2	0	0	
	T5 Sb#1	205 ± 11	189 ± 14	
	T5 Sb#2	211 ± 6	197 ± 25	

[1]In transgenic and sister lines, generation (T) is indicated
[2]natural powdery mildew infection
[3]artificial powdery mildew infection

Table 3 Morphological traits of transgenic *Pm3b* lines (mean ± SE).

Line	Chlorophyll content[1]	Plant height (cm)[2]				Seed set (%)[4]
		Natural	Artificial	Fungicide	Pooled[3]	
Bobwhite	41.6 ± 0.5	93 ± 2	92 ± 1	97 ± 1	94 ± 1	93 ± 1
Pm3b#1	41.3 ± 1.1	88 ± 0*	93 ± 2	90 ± 2	90 ± 1	95 ± 1
Sb#1	42.6 ± 0.3	92 ± 2	92 ± 2	94 ± 1	93 ± 1	94 ± 1
Pm3b#2	34.4 ± 0.8***	88 ± 1**	87 ± 1	88 ± 1**	87 ± 1***	87 ± 3
Sb#2	40.2 ± 0.4	94 ± 2	91 ± 1	96 ± 2	94 ± 1	94 ± 1
Pm3b#3	36.3 ± 1.1*	92 ± 2	89 ± 1	92 ± 2	91 ± 1	95 ± 1
Sb#3	39.6 ± 0.6	93 ± 1	91 ± 1	94 ± 2	93 ± 1	93 ± 1
Pm3b#4	38.0 ± 0.3	n.d.	n.d.	n.d.	n.d.	81 ± 2*
Sb#4	38.8 ± 0.7	n.d.	n.d.	n.d.	n.d.	94 ± 1

[1]data from microplots 2008, fungicide treatment
[2]data from microplots 2009
[3]overall mean ± SE was calculated from pooled data of the three treatments
[4]data from microplots 2008; data from artificial infection and fungicide treatment were pooled
The transgenic *Pm3b* lines were compared with their corresponding sister lines, and differences within each pair were tested for significance by Student's T-tests (chlorophyll content) or multiple linear regressions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$); SE, standard error; n.d., not determined

Table 4 Sequence and concentration of primers and probes used for RT-qPCR experiments.

Target gene (UniGene)	GenBank accession number	Amplicon length	Primer/probe	Sequence (5'-3'; modifications)	Conc (nM)[1]
Ta.31015	AY325736	108 bp	Sbi342	TGGGCAGCATCAAACGC	600
(*Pm3b*)			SuB8	CCGCTCACGGACTAGCCTC	600
			probePm3#2	6-FAM-TGCCCGTTATGAAGTAA-MGBNFQ	250
Ta.2776	AY059462	127 bp	RLILP_#1F2	GCTCTCTGTCGTTGAGGGTGA	900
			RLILP_#1R2	TTCCTTCCATGGTATCTGGCTT	300
			Probe_RLILP#1	6-FAM-CCCGGCCAGCATT-MGBNFQ	200
Ta.54227	EU267938	72 bp	CDCP_#3F2	CAAATACGCCATCAGGGAGAA	900
			CDCP_#3R2	GCTTCAGGGTTGTCCTTCCTC	300
			Probe_CDCP#3	NED-CTCTCGATGTCCTTCTC-MGBNFQ	250
Ta.30768	AF251217	81 bp	GAPDH-rt-F	TTAGACTTGCGAAGCCAGCA	900
(*GAPDH*)			GAPDH-rt-R	AAATGCCCTTGAGGTTTCCC	300

[1]Primer/probe concentration in RT-qPCR reaction.

Figure legends

Figure 1 Transgenic *Pm3b* lines Pm3b#1 to Pm3b#4 confer resistance to powdery mildew. Segments of primary leaves were infected with powdery mildew strain 07296 that is avirulent (a) or 96.10 that is virulent (b) on the *Pm3b* donor lines Chul and Chul/8*Chancellor. Cultivars Bobwhite and Chancellor were used as susceptible controls.

Figure 2 Relative *Pm3b* expression level was determined by RT-qPCR.

(a) Relative *Pm3b* mRNA levels in transgenic lines Pm3b#1 to #4 in macroplots 2008. Results are plotted as the ratio to Pm3b#1 expression at the stem elongation stage. Values report the average of 4 biological replicates and error bars the standard error of the mean (SEM). (b) *Pm3b* expression level in the *Pm3b* donor line Chul and the transgenic lines Pm3b#1 to #3 in microplots 2009. Leaves were sampled at the stem elongation stage (BBCH 32/33). Results are plotted as the ratio to Pm3b#1 with natural infection and values report the mean of three biological replicates ± SEM.

Figure 3 A fraction of the Pm3b#3 plants show transgene silencing.

(a) *Pm3b* transgene expression levels of field-grown plants at the stem elongation stage (BBCH 32) were determined by RT-qPCR in seven individual Pm3b#1 and 20 Pm3b#3 plants. Values represent the mean of three technical replicates and are plotted as the ratio to the mean of Pm3b#1. (b) *Pm3b* mRNA abundance in Pm3b#3 plants is highly variable and correlates with the level of powdery mildew resistance. Pm3b#3 seedlings were grown in a climate chamber and the first leaf was infected with powdery mildew. Seedlings were classified as resistant (R), intermediate resistant (IR) and

susceptible (S). Relative expression values depict the mean of five plants of each class plotted as ratio to Chul/8*Chancellor, and error bars the SEM.

Figure 4 Some of the transgenic Pm3b lines show pleiotropic phenotypes under field conditions.

(a) Pm3b#2 and a fraction of the Pm3b#3 plants showed chlorotic leaves when grown in the field. Representative pictures of the first leaf below the flag leaf were taken at the ear emergence stage (BBCH 59). (b) Pm3b#2 and Pm3b#4 showed open florets in some environments.

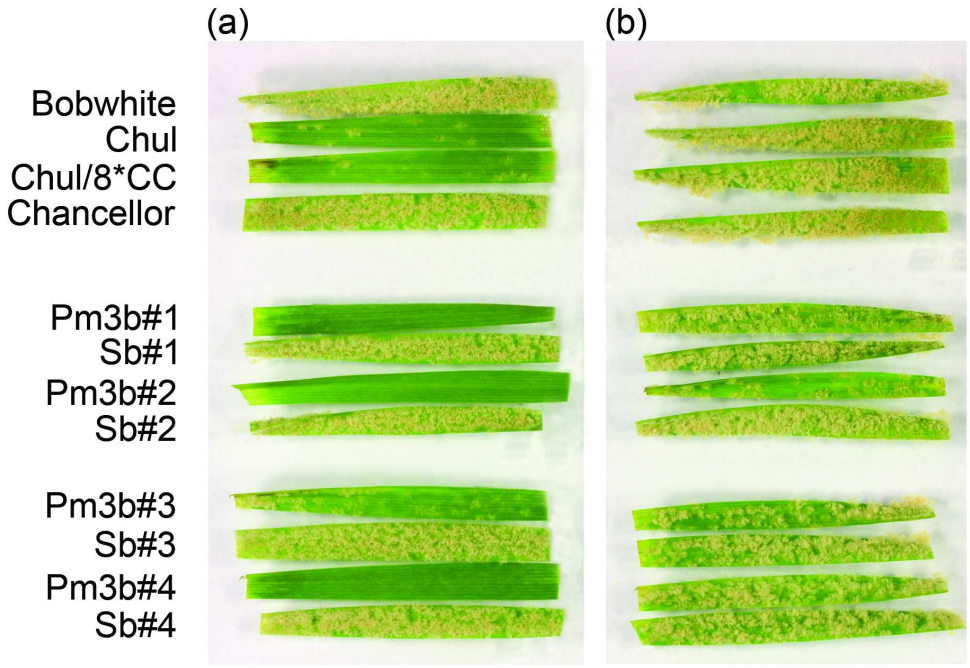

Figure 1 Transgenic Pm3b lines Pm3b#1 to Pm3b#4 confer resistance to powdery mildew.
80x54mm (600 x 600 DPI)

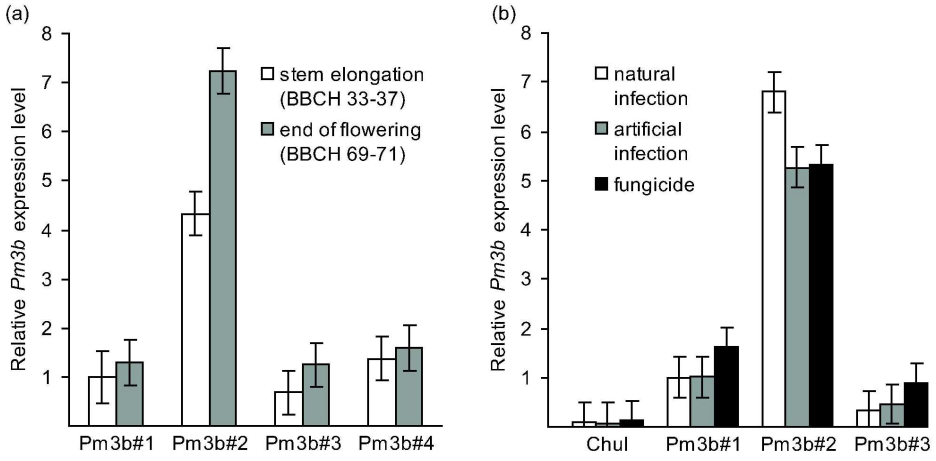

Figure 2 Relative Pm3b expression level was determined by RT-qPCR.
179x97mm (600 x 600 DPI)

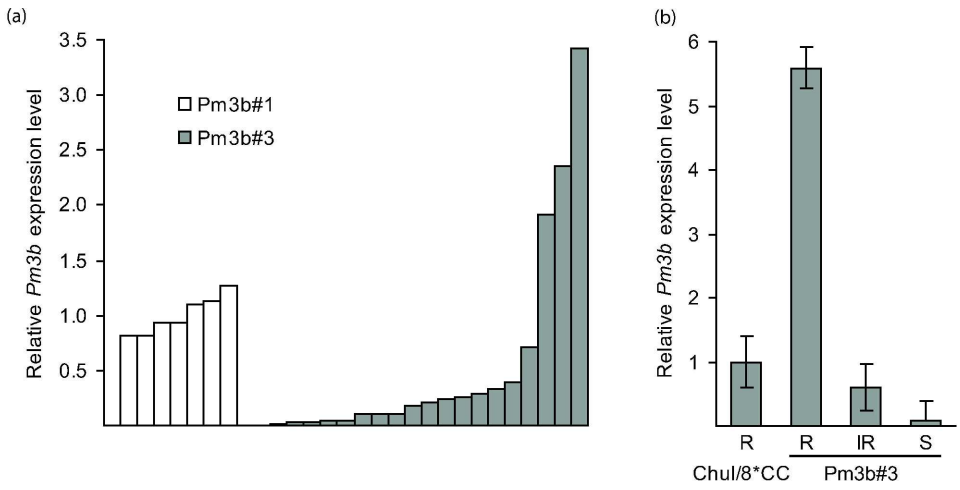

Figure 3 A fraction of the Pm3b#3 plants show transgene silencing
176x94mm (600 x 600 DPI)

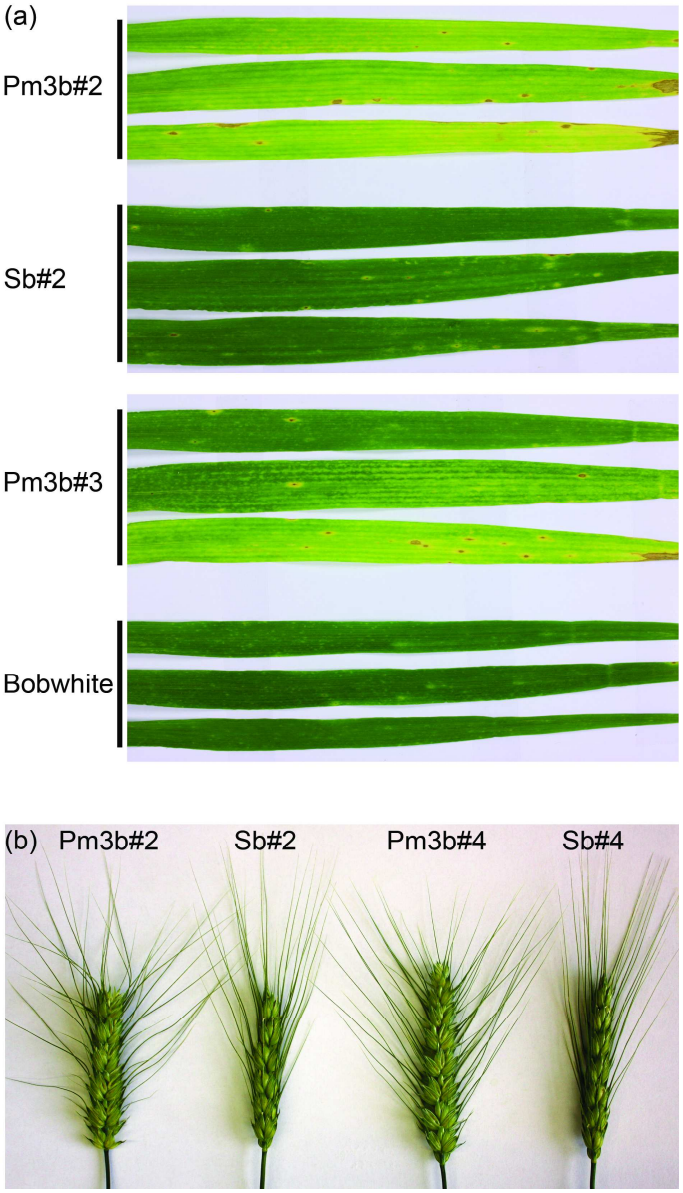

Figure 4 Some of the transgenic Pm3b lines show pleiotropic phenotypes under field conditions.
80x141mm (600 x 600 DPI)

Table S1 Powdery mildew susceptibility analysis of Pm3b#3 seedlings by leaf segment infection tests.

Generation	Analysed plants	Susceptible[1]
T_3	321	28%
T_4	351	20%
T_5	738	30%
T_6[2]	194	44%
T_7[2]	192	65%

[1]If more than 20% of the leaf surface was diseased, plants were scored susceptible.
[2]T_3-T_5 plants were derived from the powdery mildew resistant proportion of their parental generation, while the parents of the T_6 and T_7 plants were not selected.

Table S2 Morphological traits of transgenic *Pm3b* lines (mean ± SE).

	Plant height (cm)[1]		Seed set (%)[2]	
Wheat line	2008	2010	2009	2010
Bobwhite	74 ± 1	86 ± 1	95 ± 2	96 ± 1
Pm3b#1	70 ± 1	84 ± 2	91 ± 2	95 ± 1
Sb#1	73 ± 2	85 ± 1	96 ± 2	96 ± 1
Pm3b#2	68 ± 1***	79 ± 1***	91 ± 1	92 ± 3
Sb#2	73 ± 1	86 ± 1	95 ± 1	95 ± 2
Pm3b#3	73 ± 1	n.d.	96 ± 1	97 ± 1
Sb#3	71 ± 1	n.d.	94 ± 1	93 ± 1
Pm3b#4	71 ± 1	n.d.	82 ± 1***	81 ± 4***
Sb#4	75 ± 1	n.d.	97 ± 2	95 ± 0

[1]Data from microplots under natural and artificial powdery mildew infection and under fungicide treatment of each season were pooled
[2]Measurements were performed in macroplots
The transgenic *Pm3b* lines were compared with their corresponding sister lines and differences within each pair were tested for significance by multiple linear regressions (***, $P < 0.001$); SE, standard error; n.d., not determined

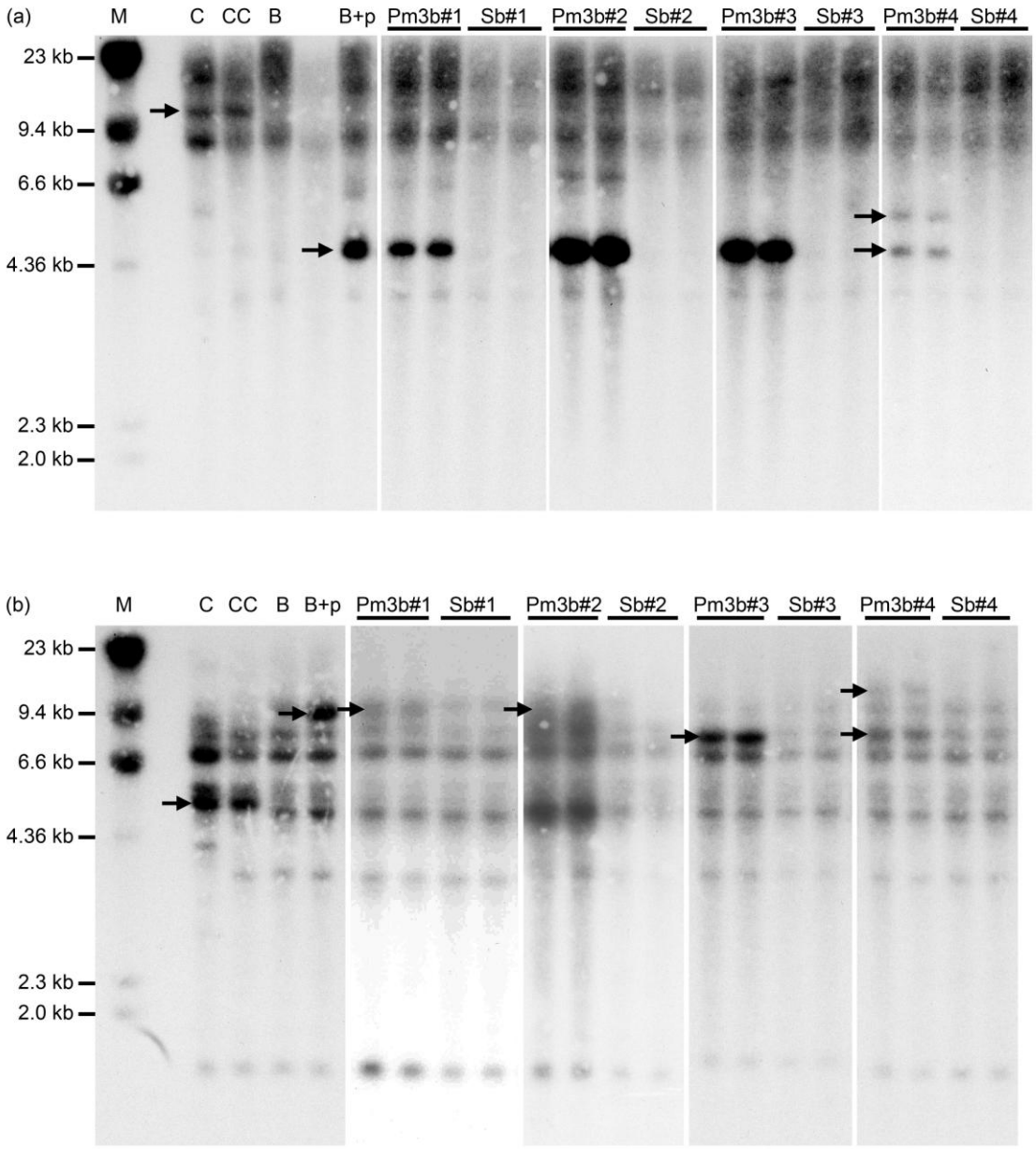

Figure S1 Southern Blot analysis of Pm3b#1-4 and Sb#1-4. Genomic DNA was digested with *Bam*HI (a) and *Sac*I (b). The blots were hybridised with probe SuB3 that binds to the PM3B encoding sequence. As positive controls, we used Chul (C), Chul/8*Chancellor (CC) and genomic DNA of Bobwhite together with plasmid DNA containing the transformed *Pm3b* gene cassette (B+p). Bobwhite (B) served as negative control. Arrows indicate *Pm3b*-specific bands. The fragment lengths of the size marker (M) are indicated on the left.

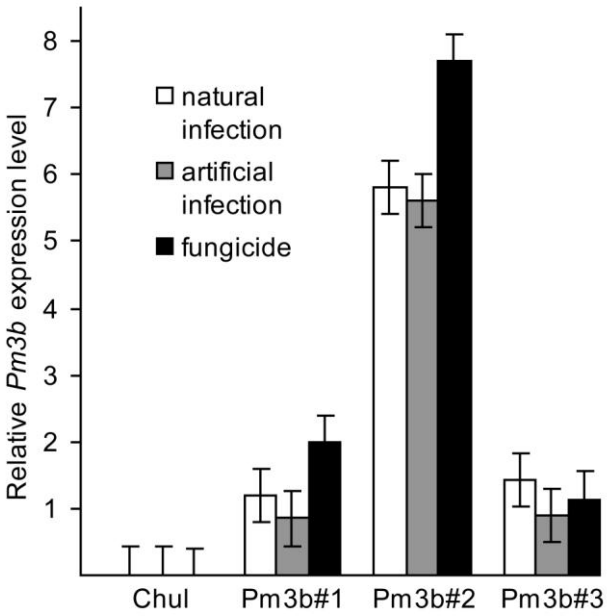

Figure S2 Relative *Pm3b* mRNA levels in landrace Chul and transgenic *Pm3b* lines at flowering stage in microplots in 2009. Leaves were collected during the flowering period (BBCH 61-65). Results are plotted as the ratio to Pm3b#1 under natural infection at stem elongation stage (Figure 2b) and values report the mean of three biological replicates ± SEM.